SUBSTRATE PARAMETERS AFFECTING PROPAGATION OF JUVENILE FRESHWATER PEARL MUSSELS *MARGARITIFERA MARGARITIFERA* (BIVALVIA: MARGARITIFERIDAE)

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*Abstract Interstitial habitat conditions are of critical importance to species inhabiting the hyporheic zone, particularly for moderately immobile species incapable of escaping poor habitat conditions. The endangered freshwater pearl mussel (*Margaritifera margaritifera *Linnaeus, 1758) has seen increasing propagation effort over the last three decades, often with mixed success. This study aimed to investigate parameters with the potential to affect juvenile survival in captivity by considering a range of habitat conditions within the substrate of a previously described propagation system using different substrate size classes (0.25–1 and 1–2mm) and cleaning regimes (weekly and monthly). Juvenile survival was highest in larger substrates, likely because of higher flow through larger pore spaces. This provided higher dissolved oxygen delivery in 1–2mm substrates cleaned weekly (8.26±0.19 mg/L) and monthly (8.24±0.44 mg/L), compared with 0.25–1mm substrates cleaned weekly (7.98±0.44 mg/L) and monthly (6.78±1.27 mg/L). The amount of organic material trapped in the substrate did not differ between treatments but the high concentrations of inorganic phosphorus liberated from ashed organic matter indicated phosphorus storage in phytoplankton. High dissolved oxygen concentrations and good water replacement between the water column and the substrate are crucial for survival in captive freshwater pearl mussels.*

Key words Interstitial, propagation, Margaritifera*, dissolved oxygen, substrate.*

INTRODUCTION

The freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus, 1758) is endangered (Moorkens *et al*., 2017) and global populations declined by over 90% during the last century (Bauer *et al*., 1980). Pearl mussels are target species of conservation (Geist, 2010), with many propagation programmes in place in various European countries (Gum *et al*., 2011). *Margaritifera margaritifera* requires pristine habitat conditions with high dissolved oxygen (DO) concentrations. The most significant factor leading to population declines in Europe is habitat degradation through siltation and increased nutrient loading (Buddensiek *et al*., 1993; Moorkens & Killeen, 2014; Santos *et al*., 2015). Juvenile mussels are particularly vulnerable to poor habitat conditions because they inhabit gravel interstices (Skinner, Young & Hastie, 2003) which can become clogged by fine particles (Brim Box *et al*., 1999; Buddensiek, 1995; Geist & Auerswald, 2007). Substrates may subsequently become anoxic leading to increased juvenile mussel mortality due to a combination of factors including direct smothering, decreasing water column-interstitial water exchange, and decomposition of organic matter (Patzner & Müller, 2001; Buss *et al*., 2009). Although the exact cause of juvenile mussel mortality has not yet been identified (Quinlan *et al*., 2014), the likely cause of death will be one, or a combination of effects, including asphyxiation, toxic effects (such as lethal levels of ammonia), or a depletion of glycogen reserves through the inability to feed effectively, followed by wasting of body tissue (Naimo *et al*., 1998; Quinlan *et al*., 2015; Beggel *et al*., 2017; Moorkens & Killeen, 2018). Juvenile mussels are intolerant of anoxia (Dimock & Wright, 1993), so sub-optimal interstitial conditions may encourage juveniles to seek higher oxygen concentrations by moving to the substrate surface where the exchange with the water column is higher (Moorkens, 2011). This puts juveniles at risk of being washed away during high flows, leading to increased risk of mortality (Sparks & Strayer, 1998).

In captive or laboratory settings, parameters such as flow (rate and direction), fine particle inputs, juvenile mussel diet, substrate characteristics and disturbance can be controlled and monitored to achieve improved survival

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and/or growth of target species. Whilst many culture facilities adopt methods which have some natural components (Beaty & Neves, 2004; Buddensiek, 1995; Hruška, 1999; Lavictoire, Sweeting, & Benito, 2014; Lavictoire *et al.*, 2016; Mummert, 2001; Preston, Keys, & Roberts, 2007), others have adopted methods which promote accelerated growth in systems and do not require water flow and/or sediment at all (Barnhart, 2006; Eybe *et al.* 2013; Lange, 2005). This brings into question which parameters truly limit juvenile growth and survival in culture and where propagation programmes should focus efforts in order to optimise survival. Gum *et al.* (2011) provides a succinct synopsis of captive rearing methods and success for a range of European and North American freshwater mussel species; it is suggested an additional review along these lines would be useful in the near future.

A number of studies have attempted to characterise pearl mussel habitat and typical nutrient concentrations that support wild populations (Bauer & Zwölfer, 1988; Buddensiek *et al.*, 1993; Geist & Auerswald, 2007; Moorkens, 2006) but the suggested targets/limits often refer to the water column and not the interstitial layer where juveniles reside (Scheder *et al.*, 2015). One of the key parameters limiting juvenile mussel survival is DO concentration (Moorkens, 2011; Quinlan *et al.*, 2014). Recommended limits of many abiotic parameters have been suggested based upon water column, rather than interstitial measurements; lethal and sub-lethal dissolved oxygen concentrations for *M. margaritifera* are not known. Dissolved oxygen concentrations can have high spatial and temporal variation even within dense mussel beds (Quinlan *et al*. 2014). Many factors affect interstitial DO including flow rate, breakdown of organic matter or waste products and exchange of interstitial water with the water column. In addition, un-ionized ammonia and nitrite can be toxic to aquatic organisms (Patzner & Müller, 2001; British Standards Institution, 2017) so poor flow through substrates may also contribute to juvenile mussel mortality through accumulation of these compounds. In controlled environments such as propagation systems, much of this habitat complexity can be removed or simplified, and it is possible to measure the effects of individual parameters on juvenile survival and growth.

There is a lack of basic knowledge regarding the physical, chemical and biological requirements of juvenile mussels and why these are important for survival. This study aimed to examine some of the habitat parameters which may contribute to differences in survival and growth of juvenile *M. margaritifera* in order to provide additional information on the habitat requirements of newly-excysted juvenile mussels. We predict that coarser substrates will provide higher flow, leading to higher dissolved oxygen concentrations and faster removal of potentially toxic compounds like ammonia. This should result in increased growth and survival of juveniles.

Materials and Methods

Experimental set-up

All experimental work took place at the Freshwater Biological Association (FBA) in Windermere, Cumbria and all experimental animals were propagated at this facility. The rearing system used is the same as described in Lavictoire *et al.* (2016) but is described briefly here. An aquarium (62×31×31cm) was set up with upper and lower chambers separated by a styrene sheet. Twelve square holes were cut into the styrene sheet and *Artemia* sieves (Hobby, Germany) with a mesh size of 0.9mm were fixed to form a water-tight seal. *Artemia* sieves with a mesh size of 0.18mm were filled with substrate (see below) and clipped in to the fixed sieves. Finally, 0.3mm mesh sieves were clipped on top of experimental sieves to retain juveniles when added to the substrate. Before addition to the system, substrate (graded to either 0.25–1mm or 1–2mm) was burned to ash organic matter (550°C for 4 hours; Carbolite 301 muffle furnace, UK) and 40g (± 0.01g) added to experimental sieves (six of each substrate size clast); substrate depth was approximately 1cm. Substrate was then exposed to running lake water for at least 21 days before addition of juveniles to allow the establishment of a stable biofilm, as in Lavictoire *et al*. (2016). All substrates were cleaned by elutriation before addition of juveniles.

Juvenile mussels

Juvenile mussels were propagated at the FBA Freshwater Pearl Mussel Ark. In summer 2014, 0+ Atlantic salmon (*Salmo salar*) were encysted with glochidia from a population of *Margaritifera margaritifera* originating from Cumbria, UK

(population details available upon request from the corresponding author). Fish were maintained at the facility until juvenile excystment the following year. In mid-July 2015, 100 newly-excysted juveniles were transferred to each experimental sieve. Thirty randomly selected individuals from each sieve were measured (length and height) to obtain initial size. A hole was punched through the mesh of each top sieve (0.3mm mesh), through which 6mm aquarium tubing was inserted. The tip rested approximately half way into the substrate so that water samples could be extracted from within the substrate. Mesh (0.2mm) covered the tubing end to avoid removal of juvenile mussels when water samples were taken. Three sieves also had a second hole so DO loggers could be inserted into the substrate. Following the cleaning method described in Lavictoire *et al.* (2016), half of the sieves were cleaned weekly and half cleaned monthly. There were three replicates of each treatment; 0.25–1mm substrate cleaned weekly, 0.25–1mm cleaned monthly, 1–2mm cleaned weekly and 1–2mm cleaned monthly. DO loggers were removed from sieves and data downloaded before any cleaning activity.

A multi-parameter sonde (Troll 9500, In-Situ, USA) was suspended in the water column in the top chamber of the aquarium to measure conductivity, DO, pH, redox potential, temperature and turbidity of the incoming water every 15 minutes for the duration of the experiment. DO loggers were introduced to the system at the beginning of week 5 (day 31) and logged at the same 15 minute intervals. Dissolved oxygen loggers used during this experiment were a DO dipping optode with Fibox 4 logger (PreSens, Germany), and two Hobo DO loggers (Onset, USA). The Hobo loggers were deployed in sieves cleaned monthly (undisturbed throughout the entire month) and the PreSens DO optode was used to log DO in treatments cleaned weekly (optode was moved at time of substrate cleaning). Upon termination of the experiment on day 57, the 0.25–1 and 1–2mm monthly sieves were cleaned and the Hobo loggers reinserted until the following day while sampling of the remaining sieves took place. This was to ascertain if cleaning increased DO concentration in the monthly treatments following a cleaning event. DO sensors were calibrated following manufacturer's instructions and measured to within an average of 0.32 mg/L and 0.08°C of each other.

Flow and organic content analysis

Upon termination of the experiment, flow rate through individual sieves was measured. Without disturbing the substrate, sieves were removed from the aquarium and placed over an empty bucket. The time taken for 1 L of water to flow through the substrate was recorded. The substrate was then examined to record the number of live juveniles and the measure the length and height of 30 individuals (randomly selected). During juvenile processing, all organic material trapped within the substrate was collected by washing substrate and equipment with distilled water. These samples were then ashed, Loss on ignition was calculated (see below), and samples were analysed for total phosphorus (TP).

The effective pore space of substrate was measured after juveniles were removed and substrate had been elutriated to remove organic matter. Substrate was placed into a measuring cylinder and water added until the meniscus rested on the substrate surface. Water was then drained into another measuring cylinder and the volume recorded. Substrate was transferred to crucibles and dried at 105°C overnight. The dry samples were weighed before being heated to 550°C for 4 hours. The burned mass of each sample was recorded once crucibles had cooled to room temperature. Empty crucibles were also weighed. Loss on ignition (LOI) was calculated using the following equation:

$$\textbf{Loss on ignition} = ((DS - AS) / (DS - DC)) \times 100$$

Where: DS=mass (g) of the crucible containing the dried sample; AS=mass (g) of the crucible containing the ignited sample; DC=mass (g) of the empty crucible.

Chemical analyses

Ammonia

On day 29, before sieves were disturbed for cleaning, a 2 ml water sample was extracted from the substrate in each sieve and three samples taken from random points in the water column. Initially, 1 ml was siphoned out of the tubes and discarded to displace water already present in the tube before the substrate sample was taken.

Ammonia-free water was prepared in advance using Amberlite IR 120 resin (Na^+ form, Aldrich Chemistry). The phenate method for

determination of ammonia concentration was used (Greenberg, 1985). Standards of ammonium chloride were prepared to concentrations of 0.00625, 0.0125 and 0.025 µg/ml. A blank (ammonia-free water) was also used to check for potential sample contamination. The blank and standard solutions were used to create a calibration curve before analysis of samples using a Double-beam Cary 60 UV-VIS spectrophotometer (Agilent Technologies, USA) at 630 nm.

Total phosphorus

The amount of TP from organic matter washed from within the substrate was analysed as described in Mackereth, Heron and Talling (1978) at the end of the experiment to give the amount of TP present after one week (treatments cleaned weekly) and one month (treatments cleaned monthly). After experiment termination, the contents of each sieve were emptied into a Pyrex dish and elutriated in the same way as for juvenile cleaning to collect organic matter. Samples were poured into a measuring cylinder, allowed to settle, and the supernatant discarded. The drying and burning procedure described above using the muffle furnace was repeated for organic matter samples.

To each ash sample, 5 ml of 5N hydrochloric acid was added and left to stand for 2 hours to make P available for analysis. Samples were filtered through Whatman No.1 filters and made up to 100 ml with distilled water. A 1 ml subsample was taken from each and neutralized by adding 1 drop phenolphthalein solution and concentrated sodium hydroxide dropwise until the solution turned bright pink. These sub-samples were then made up to 20 ml with distilled water. Phosphate standard solutions were prepared with potassium dihydrogen orthophosphate to concentrations of 0 (blank), 0.02, 0.05, 0.10, 0.15, 0.20, 0.40 and 1.00 µg/mL. The blank and standard solutions were used to create a calibration curve before analysis of samples via spectrophotometry at 880 nm. Results are reported as TP (filtered) and represent the TP bound in organic material trapped within the substrate.

Data analysis

Juvenile length and height are highly correlated (Lavictoire, 2016) and so only shell length was used for analysis here. Central Limit Theorem (Elliott, 1993) was applied to assume normality where appropriate. Analysis of Variance (ANOVA) with post hoc Tukey's HSD tests were used for data relating to juvenile survival, juvenile length, mass of biofilm, mass of organic matter within the substrate, total LOI, total phosphorus in the substrate and ammonia. T-tests were used to compare interstitial space in the two different substrate sizes. Unless otherwise stated, standard deviations are provided after means.

To ensure there was no temporal autocorrelation bias in DO data, water column data were analysed using the autocorrelation and partial autocorrelation function in the statistical package R (version 3.2.2). Analysis indicated an appropriate interval of 11 data points. Every 11th data point was extracted and used for statistical analysis; spikes in DO data relating to cleaning events (see below) were removed but are included in figures and are discussed. Paired *t*-tests were performed to explore differences in DO concentrations between the water column and treatments. For these tests a Bonferroni correction for multiple tests was applied so that $\alpha=0.006$ (n=9).

RESULTS

A summary of water quality parameters for the period 23 August–15 September 2015 is provided in Table 1. Additional data were also provided by the Environment Agency (© Environment Agency

Table 1 Mean (±SD) values for water quality parameters measured by the Troll 9500 sonde and from spot samples taken by the Environment Agency from Windermere South Basin on 17 August 2015

Parameter	Mean (±SD)
TROLL 9500 SONDE	
Temperature (°C)	16.62±0.39
Turbidity (FNU)	4.21±3.68
Redox potential (Volts)	0.53±0.02
pH	7.28±0.09
Dissolved oxygen (mg/L)	8.57±0.25
Dissolved oxygen (% saturation)	88.42±2.96
Conductivity (µS/cm)	56.06±2.13
ENVIRONMENT AGENCY DATA	
Phosphorus–P (mg/L)	0.02
Nitrogen–N (mg/L)	0.71
Filtered orthophosphate (mg/L)	0.002
Nitrogen-oxidised filtered (mg/L)	0.18
Ammonia filtered (mg/L)	0.002

and database right) for Windermere South Basin (grid ref: SD 38230 91552). All parameters appear to be within expected ranges for the species and are typical for this facility (FBA, unpublished data). Particularly important parameters to note are the low turbidity and conductivity measurements coupled with consistently high dissolved oxygen concentrations, even at temperatures toward the upper end of the acceptable thermal spectrum for *Margaritifera* (average 16.62°C during the experimental period). We report average redox readings in the open water of 0.53 V which matches open water readings reported by Geist & Auerswald (2007). The substrate layer within the rearing system was too shallow to attempt measuring the difference between open water and interstitial redox potential as has been the increasing practice in pearl mussel rivers in Europe. Orthophosphate and ammonia concentrations from the August spot sample were checked to ensure they fell within acceptable ranges for *Margaritifera*, according to the recently published CEN Standard (British Standards Institution, 2017).

Survival & size

Survival ranged from 65–87% and differed significantly between treatments ($F_{(3,8)}$=4.713; P=0.035). Highest survival was observed in the 1–2mm monthly treatment (81±8), followed by the 1–2mm weekly (80±4), 0.25–1mm weekly (72±2) and 0.25–1mm monthly treatments (68±4). Despite this significant result, *post hoc* tests could find no significant difference between individual treatments, although the difference between the 0.25–1mm monthly and 1–2mm monthly treatments was almost significant (P=0.053). This may be due to low replicate number meaning insufficient power in *post hoc* tests. Survival was strongly associated with interstitial space (ml), dissolved oxygen concentration (%), biofilm biomass (g) and flow time (mins); Fig. 1a-d. In contrast, survival did not have strong associations with interstitial organic matter (g), ammonia concentration (mg/L) or phosphorus concentration (mg/L), Fig. 1e-g.

Juvenile length was not significantly different between sieves ($F_{(11,348)}$=1.366, P=0.187) at the beginning of the experiment (mean length= 0.45±0.05mm) and was also statistically the same ($F_{(3,356)}$=0.744; P=0.526) between treatments upon experiment termination (mean=0.83±0.09mm).

Dissolved oxygen

There was a strong association between survival and dissolved oxygen concentration (Fig. 1b). Fig. 2 shows DO concentrations logged at 15 minute intervals for all treatments in mg/L and % saturation. Sonde malfunction resulted in loss of data for water column parameters between 17–23 August. In addition, DO concentration did not regain the pre-cleaning level in the 0.25–1mm monthly treatment after the first cleaning event on 23 August 2015. There was no apparent reason for a step-change in DO concentration on 25 August 2015 and as such, data between 10:00 on 23 August and 12:30 on 25 August 2015 were excluded from statistical analyses for this treatment (though data are still shown in Fig. 2).

Severe low DO spikes were recorded in all treatments when sieves were removed for cleaning (see spikes on 23 & 30 August and 6 September– Fig. 2); as low as 0.07 mg/L (<1% saturation) in the 0.25–1mm monthly treatment on 23 August 2015. These spikes are likely due to an absence of flow through the substrate when a sieve was removed for cleaning. Flow to the water column DO sensor was unaffected by sieve removal and shows stable, high DO concentrations.

Water column DO concentrations were consistently and significantly higher than any substrate measurements (Fig. 2 and Table 2), never dropping below 7.94 mg/L (81% saturation) and displayed a synchronous pattern with water temperature. DO concentrations in 1–2mm substrates were consistently high and showed no obvious decline over time (Fig. 2). Whilst always lower than the water column DO, interstitial water in the 1–2mm monthly treatment tracked the same pattern of DO fluctuations compared with the water column. Low DO spikes in the 1–2mm monthly treatment caused by weekly sieve removal were smaller in magnitude compared with the 0.25–1mm monthly treatment. DO concentration in the 0.25–1mm monthly treatment began to drop approximately 11 days post-cleaning (28 August) and fluctuated around 5.0–6.5 mg/L (51–66% saturation) from approximately day 15 to day 29 post-cleaning (1–14 September 2015). This compares with concentrations of around 8.0 mg/L (82% saturation) in the 1–2mm monthly substrate over the same period. The only instances when any treatment had a higher DO concentration than the 1–2mm monthly treatment was when the 0.25–1mm

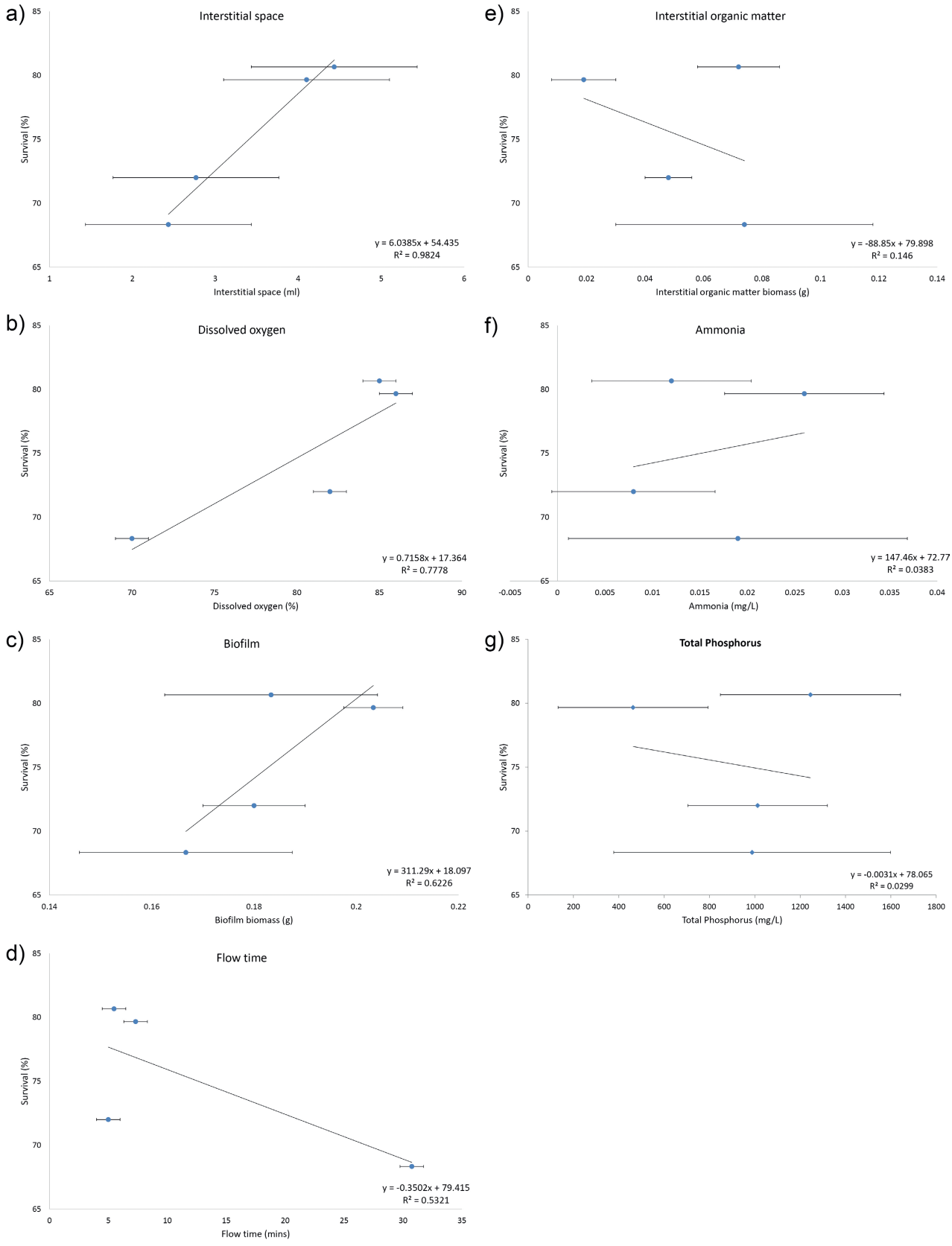

Figure 1 Mean survival (%) against mean a) interstitial space (ml), b) dissolved oxygen (%), c) biofilm biomass (g), d) flow time (mins), e) interstitial organic matter biomass (g), f) ammonia (mg/L), g) total phosphorus (mg/L). Note, all of the strong associations are provided on the left hand side and all the weak associations in the right hand side in descending order.

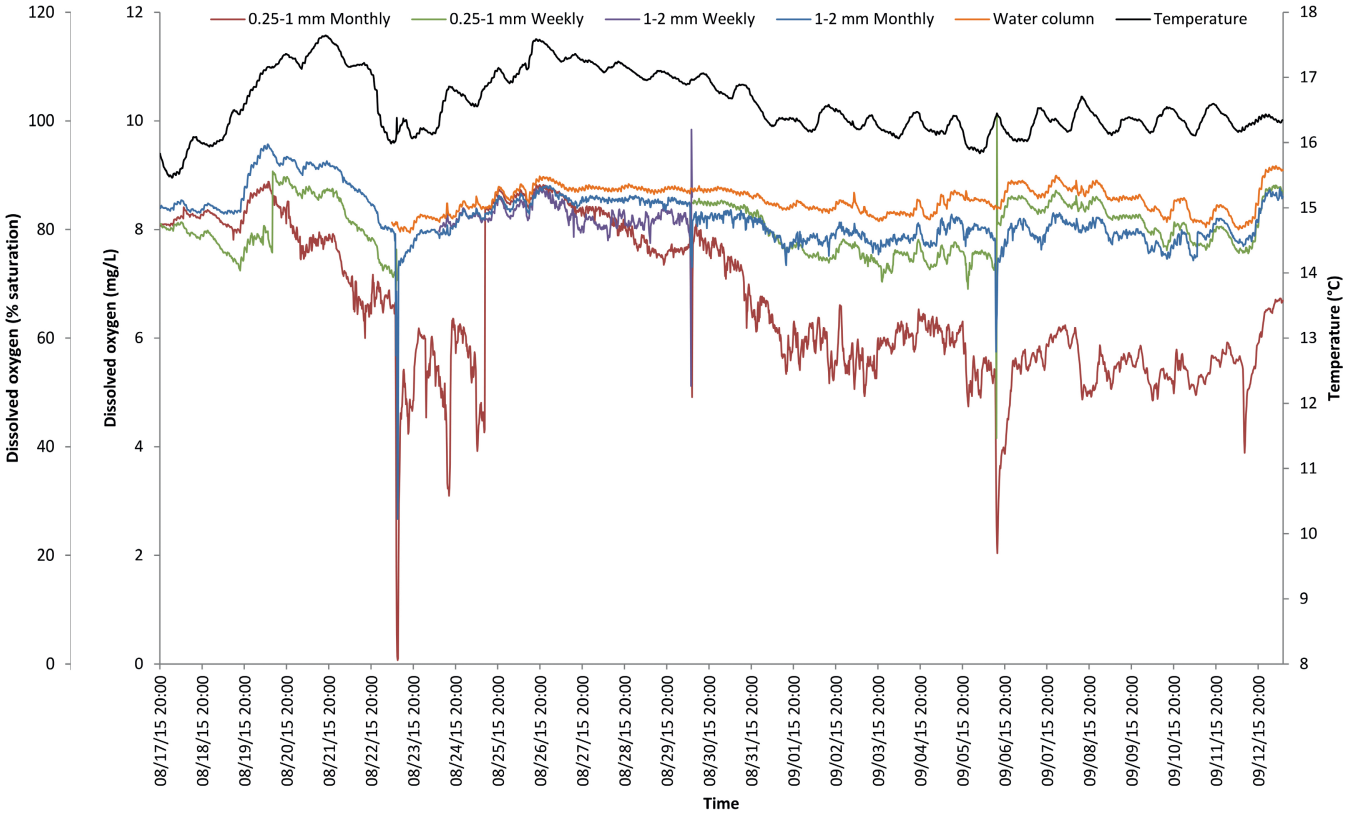

Figure 2 Time series graph showing dissolved oxygen concentration (mg/L) and saturation (%) in all treatments and the water column. Temperature (°C) also provided.

Table 2 Summary of dissolved oxygen data in mg/L and % saturation (± SD) with DO spikes identified as anomalies from cleaning events removed from analysis. N.B. Range includes low spike value in parentheses

	DO mg/L		DO % saturation	
Treatment	Mean (±SD)	Range (spike)	Mean (±SD)	Range (spike)
Water column	8.55 (± 0.25)	7.94–9.17 (N/A)	88 (± 3)	81–95 (N/A)
0.25–1mm weekly	7.98 (± 0.44)	6.90–8.81 (1.54)	82 (± 5)	70–100 (16)
0.25–1mm monthly	6.78 (± 1.27)	2.04–8.88 (0.07)	70 (± 14)	21–93 (1)
1–2mm weekly	8.26 (± 0.19)	7.75–8.76 (5.12)	86 (± 2)	81–92 (53)
1–2mm monthly	8.24 (± 0.44)	7.13–9.57 (2.67)	85 (± 5)	73–100 (27)

weekly treatment had been cleaned and this only lasted for around 4 days before DO decreased.

Upon experiment termination, the probes in the 0.25–1mm and 1–2mm monthly sieves were reinserted post-cleaning in order to assess the effect of cleaning on DO concentrations (Fig. 3). In the 1–2mm treatment DO concentration increased slightly after cleaning from around 8.5 mg/L to 8.8 mg/L. The increase in the 0.25–1mm treatment was more substantial however, increasing from around 6.5 to 8.5 mg/L. The same pattern was observed in the 0.25–1mm weekly treatment after the cleaning event on 06 September 2015 when DO increased from approximately 7.3 mg/L to 8.1 mg/L post-cleaning (Fig. 2). As Fig. 3 shows, DO concentration in the 0.25–1mm monthly treatment was more affected by sieve removal from the system than the 1–2mm monthly treatment, leading to spikes of reduced DO concentrations when sieves were absent from the system (denoted by asterisks in Fig. 3).

Interstitial space and flow

Interstitial space and flow were both strongly associated with survival (Fig. 1). Smaller

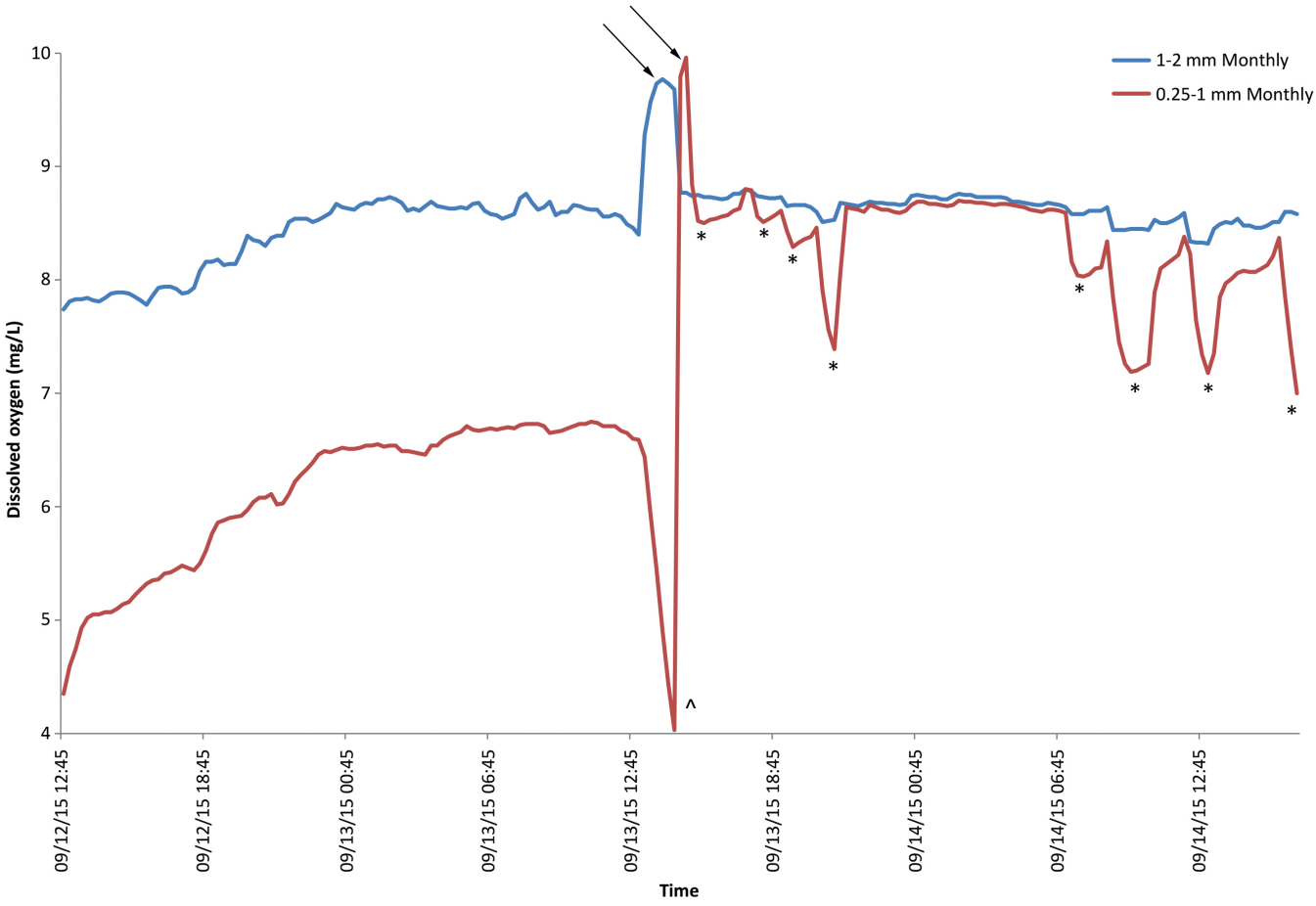

Figure 3 Time series graph showing dissolved oxygen (mg/L) in the 0.25–1mm monthly & 1–2mm monthly treatments 24 hours before and 24 hours after cleaning. Sensors were exposed to air during cleaning (arrows); low DO spike (^) is due to 1–2mm monthly sieve being removed from aquarium; spikes denoted by (*) were when other sieves were removed for sampling showing that the 0.25–1mm substrate is affected more by removal of flow (and therefore DO) when sieves are removed from the system compared with the 1–2mm monthly substrate.

substrates (0.25–1mm) had significantly less interstitial space ($t_{(10)}$=-4.725, P=0.001; 2.6±0.61 ml) compared with 1–2mm substrates (4.27±0.25 ml). Flow through undisturbed (dirty) sieves exposed to the different cleaning regimes was significantly slower through the 0.25–1mm monthly treatment compared with all other treatments (Fig 4; $F_{(3,8)}$=8.834, P=0.006) with the slowest sieve taking almost 43 minutes to clear 1 L of water. All 0.25–1mm monthly sieves had to be lightly agitated periodically to encourage water flow as it often stopped completely indicating that minimal or no flow through these sieves within the system. Once cleaned, flow through all substrates was faster with significantly faster flows through 1–2mm substrates compared with the 0.25–1mm substrates (28±5 seconds and 44±4 seconds respectively; $F_{(3,8)}$=18.802, P=0.001).

Substrate organic content and nutrient concentrations

The mass of biofilm on the substrate of different treatments was not significantly different ($F_{(3,\ 8)}$=2.756; P=0.112). Mass of organic matter within the substrate did not differ significantly between treatments ($F_{(3,8)}$=3.432, P=0.072). When combining the mass of loose organic matter and biofilm, the total LOI ranged from 0.47–0.76% (mean=0.59±0.08%). There was no significant difference in the LOI results between different treatments ($F_{(3,\ 8)}$=0.542; P=0.667).

There were no associations between survival and nutrient concentrations (Fig. 1 f & g). Inorganic TP concentrations taken from burned organic matter within the substrate were not significantly different between treatments (Table 3; $F_{(3,8)}$=2.119, P=0.176). Ammonia samples taken before cleaning on day 29 (17 August 2015)

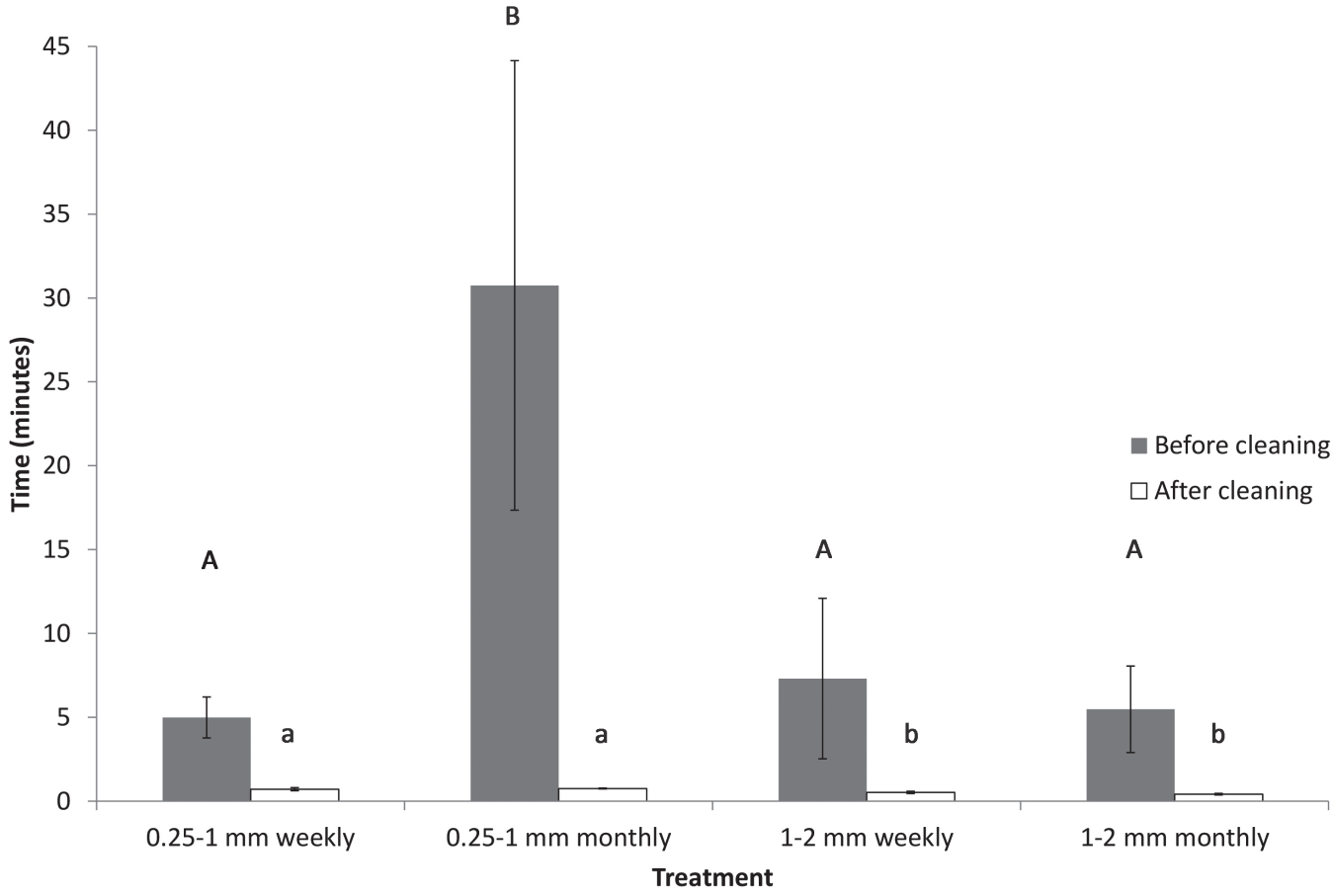

Figure 4 Bar graphs showing average time (minutes ±SD bars) for 1 L of water to flow through substrates in the different treatments before and after cleaning. Different letters over bars denote when flow was significantly different between treatments (note upper case letters used for before cleaning and lower case for after cleaning).

Table 3 Mean (± SD) concentrations of ammonia and TP

Treatment	Ammonia (mg/L)	TP (mg/L)
Water column	0.014 (± 0.007)	N/A
0.25–1mm weekly	0.008 (± 0.009)	1012.33 (± 307)
0.25–1mm monthly	0.019 (± 0.018)	998.00 (± 610)
1–2mm weekly	0.026 (± 0.008)	462.93 (± 330)
1–2mm monthly	0.012 (± 0.008)	1245.33 (± 397)

showed no significant difference between treatments or the water column (Table 3; $F_{(4, 14)}=1.384$; $P=0.307$).

DISCUSSION

The aim of this study was to identify habitat parameters capable of limiting growth and survival of juvenile freshwater pearl mussels (*Margaritifera margaritifera*) in captivity; an area outlined as requiring additional research by Geist (2010). The experimental methods used in this investigation use strict size clasts and precise cleaning intervals, neither of which would naturally occur in the wild. We have demonstrated however that, in this simplified system, even subtle differences in these parameters can significantly affect juvenile survival. Better understanding of the effects of various environmental parameters thought to be important for juvenile mussel survival will help refine propagation methods and reduce the high mortality rates typically observed in the youngest juveniles. Understanding the limits of and interactions between a wide range of environmental parameters has been identified as a priority for wild mussel populations (Boon *et al.*, 2019).

Dissolved oxygen concentration, substrate size (which governs interstitial pore space) and flow appear to be the limiting factors in the system described here (Fig. 1), with higher survival

observed in larger substrates with higher flow rates and DO concentrations. These findings echo previous findings from fish and mussel studies which show increased fine material and poor water exchange between the water column and the shallow hyporheic zone are detrimental to emergence times (fish), survival and growth (Sternecker & Geist, 2010; Denic & Geist, 2015; Duerregger *et al.*, 2018). The current work was deliberately undertaken during late summer when water temperature and primary production were at their highest; these conditions are likely to be the most challenging for juvenile *M. margaritifera* (Boon *et al.*, 2019). This also incorporates the period of highest mortality of newly-metamorphosed juveniles at both this (Lavictoire *et al.*, 2016) and other freshwater mussel rearing facilities (Buddensiek, 1995; Gatenby, Neves, & Parker, 1996; Gatenby, Parker, & Neves, 1997; Jones, Mair, & Neves, 2005; O'Beirn, Neves, & Steg, 1998).

Dissolved oxygen concentration appears to be one of the most important factors affecting survival of juvenile *M. margaritifera*, corroborating previous findings from wild populations (Geist & Auerswald, 2007; Quinlan *et al.*, 2014). Dissolved oxygen concentrations in rivers with *M. margaritifera* should be consistently high (Boon *et al.*, 2019) and this study found that treatments with the highest DO concentrations showed the highest survival. Substrate DO concentration is governed by several factors including interstitial pore space (larger pore spaces provide less friction and therefore higher flows) so it is perhaps not surprising that these two parameters both showed strong positive associations with survival (Fig. 1). The species has complex laterofrontal cirri (part of the feeding apparatus) capable of capturing very small particles (Lavictoire *et al.*, 2018) so pressures such as silt infiltration or excess algal biomass may have severe consequences for juveniles as they must expend energy removing inappropriate particles before consumption. It is also not easy to quantify substrate dissolved oxygen pressures caused by decomposing organic matter in nutrient enriched conditions. The 1–2mm monthly treatment provided the highest and most stable DO profile; an average of 8 mg/L (82% saturation) resulted in the lowest mean mortality over the experimental period of 2 months (19%±8). Within this rearing system, there was a general trend of declining DO over time in the smaller substrate treatments (0.25–1mm), likely due to reduced flow as substrates became blocked. This trend was not observed in the 1–2mm substrates with substrate DO concentrations remaining consistently high and following the same fluctuations as water column readings. Substrate DO concentration in the 0.25–1mm substrates began to decrease in as little as two days and remained lower until cleaning restored higher DO levels; fluctuations in DO were also higher than in larger substrates. Both low and fluctuating DO, if not lethal, could cause stress and may alter behaviour. The effects of low DO spikes created by interrupting flow when removing sieves from the system for cleaning were not lethal to juveniles, but in future this effect can be ameliorated by replacing the missing sieve with a 'blank' containing substrate during cleaning. This attention to detail is important in captive breeding design and management to ensure maintenance of high DO concentrations. Studies on the effect of low DO on mussel behaviour and survival/growth are particularly important in species such as *M. margaritifera* which preferentially inhabits high-DO environments and has a prolonged juvenile stage inhabiting the interstitial layer. High water column-interstitial exchange is a predictor of functional pearl mussel habitats and the presence of juvenile mussels (Buddensiek *et al.*, 1993; Geist & Auerswald, 2007). Captive rearing systems should therefore strive to achieve at least the DO concentration reported in the most suitable treatment in this investigation (82% saturation), with minima no lower than 73%. The mean value on its own has little scientific value without considering the severity and duration of the low DO spikes. The treatment which had the most pronounced low DO spikes also had the highest juvenile mortality rate so it is important to consider separating the average DO in the "steady state" situation, i.e. between cleaning events, and the lower DO spikes associated with cleaning the system. The importance of minimum DO levels has been demonstrated in the wild by Quinlan *et al.* (2014), where their study using optodes in the substrate found average DO saturation ranged between 81–103%, but minimum levels dropped as low as 0.26%.

Both the biofilm biomass and the biomass of organic matter within the substrate at the end of the experiment showed no significant difference

between treatments (grouped means (±SD) of 0.18g (0.02) and 0.05g (0.03) respectively). This indicates that the different flow conditions between treatments created by significantly different pore size did not lead to differences in organic matter within the substrates or adhered to the substrate itself. It is not known if substrate biofilm plays any dietary role for juveniles, but the statistically insignificant difference in biomass under different flow conditions implies flow was not different enough to elicit significantly different biofilm biomass through processes such as scouring. Few data exist describing the amount of organic matter juveniles can withstand within substrates and what the effect of organic matter is on survival and growth. In this study the percentage of organic matter within the substrates and as biofilm (adhered to the substrate) was comparable to that of recruiting Scottish pearl mussel rivers (Tarr, 2008), 0.47–0.76% and 0.5–1% respectively. Tarr (2008) however analysed only fractions <0.5mm compared with this study which did not discriminate on size, therefore Tarr's findings may be an under-estimate of the true organic matter content. Total phosphorus concentrations were not significantly different between treatments which is expected considering there was no significant difference in organic content between treatments. Windermere has shifted from a mesotrophic to a eutrophic lake in recent decades with a subsequent increase in the algal biomass. Whilst the concentration of phosphorus reported in Table 1 appears acceptable, the TP concentration reported here highlights that elevated P concentrations are masked through conversion to algal biomass, underlining the problem of nutrient loading in Windermere (Reynolds & Irish, 2000).

Ammonia concentrations in this investigation are lower than acutely or chronically toxic concentrations reported for North American unionids (Augspurger *et al.*, 2003; Mummert *et al.*, 2003; Wang *et al.*, 2007, 2008). It is assumed that the flow-through nature of the system prevents the accumulation of high ammonia concentrations and, whilst higher than the 0.01 mg/L concentration suggested by Moorkens (2006), the treatments do not differ significantly from the ammonia concentration in the water column and do not appear to result in high juvenile mortality in this system. The presence of biofilm on the substrate and oxic conditions may help oxidise ammonia into less harmful ions, as suggested by Eybe *et al.* (2013). Flow also affects the structure and function of biofilm communities (Battin *et al.*, 2003) which has the potential to be a source of food in early juvenile life. The species composition of biofilm and its nutritive value was outside of the scope of this study but research in this area would be beneficial to assess its potential as a food source for *M. margaritifera*.

The combined results of this study demonstrate that juvenile mussels do not need a complete loss of oxygen to die, as the treatments with relatively mild hypoxia resulted in increased mortalities. The potential in-combination effects of certain parameters on juvenile stress and ultimate survival/vigour require further investigation. For example, is the mechanism of mortality in smaller substrate sizes simply lower flow leading to lower DO concentrations and resulting stress/mortality, or is it due to lower flow (and therefore lower DO) coupled with lower delivery of suitable food particles leading to starvation?

CONCLUSIONS AND SIGNIFICANCE FOR CAPTIVE REARING PROGRAMMES

This study confirms previous findings that coarser, uncompacted substrates, lacking fine sediments and organic material, provide better interstitial environments for juvenile mussels and fish (Brim Box *et al.*, 1999; Denic & Geist, 2015 ; Duerregger *et al.*, 2018; Geist & Auerswald, 2007; Lavictoire *et al.*, 2016; Liberty, Ostby, & Neves, 2007; Quinlan *et al.*, 2014; Sternecker & Geist, 2010; Wood & Armitage, 1997). In this system 1–2mm substrate cleaned monthly provided the highest juvenile survival, with the highest flow rates and DO concentrations. It was also one of the easiest treatments to maintain, requiring cleaning only once per month with sieves taking between 3–5 minutes each to clean. All other parameters did not significantly affect juvenile survival in this flow-through system. This study has outlined several areas requiring additional investigation, particularly the oxygen requirements of juvenile and adult *M. margaritifera* including lethal/sub-lethal concentrations and the effect of low DO pulses.

There is still much we do not know about the biological requirements of juvenile *M. margaritifera* but this investigation provides some preliminary information about how fundamental factors

such as substrate size and cleaning regime (or substrate flushing in the wild) can have major impacts on a juvenile mussel's growth and survival. Ensuring high DO within the substrate should be a primary consideration for captive rearing programmes; this may involve the omission of substrate entirely (e.g. Barnhart, 2006).

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